APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1992, p. 4001–4010 Vol. 58, No. 12
0099-2240/92/124001-10$02.00/0

Isolation, Cultural Maintenance, and Taxonomy of a Sheath-Forming Strain of *Leptothrix discophora* and Characterization of Manganese-Oxidizing Activity Associated with the Sheath

DAVID EMERSON† AND WILLIAM C. GHIORSE*
Section of Microbiology, Cornell University, Ithaca, New York 14853-8101

Received 29 June 1992/Accepted 5 October 1992

***Leptothrix discophora* SP-6 was isolated from the outflow reservoir of an artificial iron seep. Its sheath-forming phenotype was maintained by slow growth in a mineral salts-vitamin-pyruvate medium under minimal aeration at 20 to 25°C. A sheathless variant, SP-6(sl), was isolated from smooth colonies that appeared on spread plates after rapid growth of SP-6 in well-aerated cultures. SP-6 and SP-6(sl) are closely related but not identical to the previously studied sheathless strain SS-1 (ATCC 43182). Increasing Mn^{2+} concentrations in the growth medium of SP-6 increased the phase density of the sheath, indicating increased Mn oxide deposition in the sheath. Electron microscopy of cultures grown without added Mn^{2+} revealed that the sheath consisted of a well-defined inner layer, 30 to 100 nm thick, and a diffuse outer capsular layer of variable thickness. Mn oxides were identified in the sheath by their characteristic ultrastructure, electron density, and X-ray-dispersive energy spectra. In heavily encrusted sheaths, the Mn oxides were evenly distributed in both layers of the sheath. Sheathed cells retained more Mn-oxidizing activity than did sheathless cells after washing with distilled, deionized water; the sheath retained some of its activity after an EDTA-lysozyme-detergent treatment which removed the cells. An ultrafiltration-dialysis procedure significantly increased the recovery of activity from spent media of SP-6 over that reported previously for SS-1 (L. F. Adams and W. C. Ghiorse, J. Bacteriol. 169:1279–1285, 1987). A 108-kDa Mn-oxidizing protein was identified in concentrated spent media of SP-6 and SP-6(sl), and the activity of the concentrates showed stability in detergents comparable to that of SS-1 and patterns of heat inactivation and chemical inhibition similar to those of SS-1.**

Sheathed iron bacteria of the genus *Leptothrix* are found commonly in aquatic and terrestrial habitats containing aerobic-anaerobic interface zones in which Fe and Mn are cycled between their oxidized (insoluble) and reduced (soluble) forms (17). These bacteria often are the dominant bacteria in such habitats, where their filamentous growth habit and Fe- and Mn-oxidizing ability can cause massive accumulation of ore-forming ferromanganese minerals or clogging of water distribution systems. The metal-oxidizing ability is a prime example of a biological mechanism for control of trace metals associated with ferromanganese minerals (15), which is important in biogeochemical cycling of the metals and may have applications in metal recovery processes (17).

Two diagnostic characteristics of the genus *Leptothrix* are formation of a sheath and deposition (oxidation) of Fe and Mn in the sheath (21, 28). However, axenic cultures of *Leptothrix* spp. tend to lose their sheath-forming capacity upon growth and repeated transfer in culture media (2, 15, 21, 28). Several recent studies on Fe and Mn oxidation by *Leptothrix discophora* (3, 4, 7, 9, 10) have employed the sheathless strain SS-1 (ATCC 43182), which was originally isolated and characterized in our laboratory (16). One problem with these studies has been the lack of a sheath-forming strain of *L. discophora* for comparison.

The extracellular Mn-oxidizing activity of SS-1 is at least partly attributable to an excreted Mn-oxidizing protein with an apparent molecular mass in the range of 105 to 110 kDa (3, 7). Recent work (9) has shown that SS-1 also excretes a 150-kDa iron-oxidizing protein. Both proteins are present in spent culture media along with a mixture of heteropolymers. The Mn-oxidizing activity recovered from spent media of SS-1 is stable in sodium dodecyl sulfate (SDS) and displays enzyme-like kinetics for Mn^{2+} oxidation; it is inhibited by heat, azide, and elevated Mn^{2+} concentrations, as well as a number of other chemical treatments known to denature proteins or inhibit enzyme activity (3, 7). The Fe-oxidizing activity also is stable in SDS, displays enzyme-like kinetics for Fe^{2+} oxidation, and is inhibited by azide, cyanide, and $HgCl_2$ (9, 10). Progress in studying the biochemistry of the metal-oxidizing activities of SS-1 has been hampered by low yields of active protein from concentrated spent media. Large-scale isolation and purification of the proteins has not been achieved.

Because of the lack of sheath-forming strains of *L. discophora* for study, we considered it important to determine procedures for isolation and maintenance of sheath-forming strains in axenic culture. In this report, we describe the isolation and cultural maintenance of strain SP-6 and its sheathless variant SP-6(sl). We also establish the taxonomic position of the new strains relative to SS-1, describe the ultrastructure of the sheath relative to Mn oxide deposition, and compare properties of the Mn-oxidizing activities of the three strains.

MATERIALS AND METHODS

Inoculum sources, isolation procedures, and culture media. Sheath-forming *L. discophora* strains were isolated from surface film and flocculent material in the outflow reservoir of an artificial iron seep donated to our laboratory by Norman C. Dondero. The construction was similar to that of

* Corresponding author.
† Present address: Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824.

flowthrough devices described by Mulder and van Veen (21). Aerated tap water was percolated through a cylindrical soil column (60 by 15 cm) containing a centrally inserted iron rod. The soil and rod served as a source of reduced Fe and Mn. The effluent dripped slowly (approximately 10 to 20 ml · h^{-1}) into a shallow, unmixed reservoir which overflowed to drain. Occasionally, kernels of corn were added to the reservoir to increase the amount of metabolizable organic matter. Many microorganisms flourished in the reservoir, but sheathed bacteria dominated in specific zones, particularly in surface films at the air-water interface, and in O_2 gradients at the periphery of black, iron sulfide-containing, sulfate-reducing zones that formed around the kernels of corn.

When sheathed bacteria were observed microscopically in brownish surface film material or in fluffy yellowish material surrounding the black iron sulfide zones, the material was streaked on seep water-Mn agar medium composed of filtered seep water plus 0.1 mM $MnSO_4$ and 1.5% agar. The mixture was autoclaved for 20 min at 15 lb/in^2. After 2 to 4 days of incubation at room temperature (20 to 23°C), flat, brown, Mn oxide-containing colonies with filamentous edges typical of *L. discophora* (15, 16) were picked and restreaked on seep water-Mn agar medium. Primary *L. discophora* colonies usually harbored other heterotrophic bacteria as well. These satellite bacteria were avoided by picking material for transfer from zones at the colony edge containing leading strands of *L. discophora* filaments under a Zeiss SV8 stereomicroscope. Clones were transferred and examined for absence of satellite bacteria at least twice before they were deemed to be axenic cultures.

MSVP medium (13) consisted of 10 mM HEPES (*N*-2-hydroxyethelpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.0, plus a mineral salts mixture and filter-sterilized vitamins (2) and 0.1% sodium pyruvate as a carbon energy source; MSVP liquid medium was used to grow the axenic isolates in broth cultures. When required, MSVP medium was solidified by adding 1.5% agar to the buffered mineral salts solution before autoclaving. When Mn was desired in solid or liquid media, it was provided after autoclaving by adding appropriate amounts of a filter-sterilized solution of 0.1 M $MnSO_4$. During the course of this work, it was found that our original source of sodium pyruvate supplied supplemental iron to the MSVP medium. Sodium pyruvate from new sources (Sigma and Aldrich) did not contain enough iron to support maximum growth of *L. discophora* isolates. Therefore, filter-sterilized ferric ammonium citrate or ferric sulfate solution was added to the MSVP medium after autoclaving to achieve a final Fe concentration of 1 ppm. This supplemental iron promoted maximum growth of the *L. discophora* isolates in MSVP medium.

One isolate, designated strain SP-6, was selected to represent several morphologically identical isolates of *L. discophora* obtained from the seep. Sheathed SP-6 cultures were maintained by growth on MSVP agar medium at room temperature (20 to 23°C); storage was on Parafilm-bound agar plates at 4°C. Stock cultures grown in liquid MSVP medium were preserved for long-term storage by adding 10% glycerol to the culture before freezing in liquid N_2.

Taxonomic studies. Sheathed *L. discophora* isolates were compared with those of the previously studied sheathless strain SS-1 (ATCC 43182) (1, 2, 16). Morphology, dimensions, and motility of individual cells were determined by phase-contrast light microscopy as previously described (2). The presence and location of flagella were determined by negative staining in an electron microscope as previously described (2). The presence of poly-β-hydroxyalkanoate granules was determined in a light microscope by their characteristic phase-dense or refractile appearance under phase-contrast optics and their orange fluorescence after staining with Nile Blue A (24). Catalase and oxidase activities, obligate aerobic growth, and growth with nitrate as an electron acceptor were determined by standard methods (25). Utilization of different carbon sources was determined by growth in the buffered mineral salts-vitamin mixture of MSVP medium (2, 13) amended with 0.1% (wt/vol) of the sodium salts of the carbon sources pyruvate, gluconate, succinate, and malate. Fumarate was added as fumaric acid. A stock solution of each carbon source was filter sterilized and added to 50 ml of liquid medium in a 125-ml Erlenmeyer flask immediately before inoculation with 1 ml of a log-phase culture. The flask was incubated at 25°C on a rotary shaker at 150 rpm. Cultures that showed visible turbidity within 5 days after each of three successive transfers were scored positive for utilization of the carbon energy source.

Determination of growth rates. Growth rates of SP-6 cultures were determined by measuring increases in A_{600} during growth in liquid medium. To minimize the effects of aggregation, 3- to 4-ml samples of the culture were expressed into a 1-cm cuvette through a 25-gauge needle mounted on a 10-ml syringe to disrupt aggregates before measurement of the A_{600} in a Bausch & Lomb Spectronic 20 spectrophotometer. Growth was also determined by direct cell counts using phase-contrast microscopy as previously described (1).

Isolation of a sheathless variant. A sheathless variant of *L. discophora* SP-6 was obtained by repeated transfer of 1 ml of a log-phase culture to 100 ml of PYG medium (1) or MSVP medium in a 250-ml flask. Incubation was at 25°C on a rotary shaker at 150 rpm. After five serial transfers at approximately 24-h intervals, loopfuls of the final culture were streaked onto plates containing MSVP agar medium amended with 0.1 mM $MnSO_4$. Raised, brown, Mn-oxidizing colonies with smooth edges were selected and transferred to MSVP liquid medium without added Mn. After 24 to 48 h of incubation with shaking at 150 rpm and 25°C, the culture was examined for absence of sheathed filaments at a magnification of ×1,000 in a Zeiss Standard 18 microscope through a 100×/1.30 oil immersion phase-contrast objective lens and matching phase-contrast condenser system. Cationic colloidal iron Prussian blue stain (26), which enhanced visibility of sheath material, was used to confirm sheath absence in presumptively sheathless cultures.

Microscopic localization of Mn oxides. The distribution of Mn oxides associated with sheathed and sheathless cells was determined in the phase-contrast microscope by examining MSVP liquid cultures amended with 0.05, 0.10, and 0.20 mM $MnSO_4$. The cultures were grown statically at 25°C for 2 to 3 days and mixed immediately before sampling. The Zeiss Standard 18 microscope with an attached Zeiss MC63 camera system was used to photograph representative fields on Kodak Plus-X film.

Mn oxides also were found in SP-6 sheaths at higher resolution by using conventional transmission electron microscopy and scanning transmission electron microscopy (STEM) coupled with energy-dispersive X-ray microprobe analysis. For electron microscopy, cultures were grown in MSVP liquid medium amended with 0.2 mM $MnSO_4$ and then they were harvested by centrifugation for 10 min at 10,000 × *g*. The cell pellets were fixed by suspension in 1.0% glutaraldehyde buffered at pH 7.0 with 0.1 M sodium cacodylate plus 10 mM $CaCl_2$ for 30 min at room temperature. Conventional transmission electron microscopy samples

were washed and osmicated by centrifugation and suspension in Ca^{2+}-amended cacodylate buffer and then in a 2% aqueous OsO_4 solution overnight at room temperature. The osmicated material was pelleted by centrifugation, enrobed in molten 2% agar, cooled, cut into small blocks, and placed in a 0.5% aqueous uranyl acetate solution for 2 h at room temperature. Dehydration was in a graded series of ethanol solutions. Embedding was in an Epon-Araldite epoxy mixture (Electron Microscopy Sciences). Thin sections (50 to 100 nm) were cut with a diamond knife on an LKB IV ultramicrotome, collected on Formvar-carbon-coated copper grids, and stained with 2% aqueous uranyl acetate and 0.3% aqueous lead citrate as previously described (2). A Philips 300 transmission electron microscope operated at an accelerating voltage of 80 kV was used. Conventional transmission electron microscopy photomicrographs were recorded on Kodak electron image film.

STEM samples were fixed only in buffered glutaraldehyde solution and then washed in Ca^{2+} cacodylate buffer as described above. The osmication and uranyl acetate steps were omitted, but dehydration and embedding were done as described above. Semithin sections (approximately 200 nm) were picked up on uncoated copper grids and then coated lightly with carbon in a Balzers BAE 80 vacuum evaporator but not stained. A Hitachi H-8010 hybrid STEM instrument with an attached Tracor-Northern X-ray microprobe analysis unit operated at 200 kV was used for STEM and energy-dispersive X-ray microprobe analysis. STEM photomicrographs and X-ray spectra were recorded on Polaroid type 55 film.

Fractionation of Mn-oxidizing activity in cultures. Mn-oxidizing activity was measured by monitoring the initial rate of increase in A_{366} during Mn oxide formation in a reaction cuvette of a Gilford 260 recording spectrophotometer as previously described (3, 19). Typically, this assay was performed by adding a measured sample volume (20 to 115 μl) containing complete cultures, culture supernatants (spent media), washed cell suspensions, or cell-free sheath suspensions to a cuvette with a capacity of 250 μl. The sample was diluted with 25 mM HEPES buffer, pH 7.3, to a total volume of 200 μl. The reaction was started by addition of 50 μl of a 250 μM $MnSO_4$ solution and mixing to achieve a final concentration of 50 μM Mn^{2+}. The reaction cuvette was compared with a control cuvette containing all constituents except the $MnSO_4$ solution, which was replaced by distilled water.

Cell-free sheaths were prepared from log-phase MSVP liquid cultures by centrifugation at 10,000 × *g* for 20 min at 4°C. The pellet was suspended in distilled, deionized water and centrifuged again to wash the cells. The final pellet was suspended in a few milliliters of lysis solution consisting of 2.5 mM EDTA plus 150 μg of lysozyme per ml. This mixture was incubated for 30 min at 37°C. *N*-Lauryl sarcosine (Sarcosyl; Sigma) was then added to a final concentration of 1%; the mixture was incubated for an additional 20 min at room temperature. The detergent-treated sheath samples were examined by cationic colloidal iron staining and phase-contrast microscopy as described above to be sure that they were free of intact cells. Cell-free sheaths were washed three times by centrifugation in distilled, deionized water and stored on ice.

Supernatants of mid-log-phase cultures were concentrated by an ultrafiltration-dialysis procedure in which approximately 150 ml of 10,000 × *g* culture supernatant was passed through an ultrafiltration unit (Pharmacia) (30,000-molecular-weight cutoff) under air pressure of 20 lb/in^2 at 4°C. Approximately 10 ml of the ultrafiltration concentrate was diluted with 50 ml of distilled, deionized water and filtered again to a final volume of approximately 5 ml. The concentrate was then placed in a short length of dialysis tubing (Spectrapor) with a 12,000- to 14,000-molecular-weight cutoff which was clamped at both ends and covered with Aquacide IV (Calbiochem) for 6 to 8 h at 4°C. The final volume of the concentrate after Aquacide IV treatment was approximately 3 ml.

The protein contents of cell suspensions and washed sheath suspensions were determined by using the microassay procedure of a Bradford-type (8) protein assay kit (Bio-Rad) with lysozyme as the standard.

Electrophoresis. Concentrated supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (3), by using the discontinuous buffer system of Laemmli (20) and 1.5-mm-thick gels in a Hoefer SE 250 gel unit (6 by 8 cm). The stacking and resolving gels contained 4 and 12.5% polyacrylamide, respectively. The electrophoresis treatment buffer contained 60 mM Bicine (pH 8.8), 30% glycerol, and 6% SDS; treatment buffer was mixed in a ratio of 1:3 with samples before they were added to sample wells. Electrophoresis was done with a Buchler 3-1500 power supply operated at 25 mA of constant current at 4°C.

For comparison of Mn-oxidizing bands with protein bands in the same sample, identical samples were loaded in lanes on two sides of a gel. After electrophoresis, one-half of the gel was stained with Coomassie blue R-250 or with a commercially available silver staining kit (Bio-Rad). The other half of the gel was washed to remove SDS and then soaked in 0.1 M $MnSO_4$ solution to reveal Mn-oxidizing bands as previously described (3).

SDS-PAGE was also done on total membrane fractions obtained by French pressure cell disruption and ultracentrifugation of log-phase cells at 48,000 × *g* as previously described (3, 11). Electrophoresis of the membrane fractions was done in 10% polyacrylamide gels by using the buffers, electrophoresis conditions, and protein staining with Coomassie blue described above.

RESULTS

Isolation and maintenance of strain SP-6. Several sheath-forming isolates of *L. discophora* were obtained from the artificial iron seep on seep water-Mn agar medium. Strain SP-6 displayed the flat, brown colonies with filamentous edges characteristic of the original SS-1 and other freshly isolated *L. discophora* strains (16). SP-6 was isolated in May 1984 and has been maintained since then as a sheath-forming culture on MSVP medium. Cultural conditions encouraging slow growth were found to be important in maintaining the sheath. We achieved this in liquid cultures by static incubation at 20 to 25°C, which limited aeration and thus slowed the growth rate. Long-term maintenance of sheathed cultures was achieved by growing SP-6 on MSVP agar plates and storing the plates at 4°C.

Isolation of sheathless variants. Sheathless variants of SP-6 were obtained by serially transferring the parent SP-6 culture in either MSVP or PYG liquid medium under increased aeration conditions (shaking at 150 rpm) and then plating the culture on Mn-containing MSVP medium. Smooth, brown colonies characteristic of the sheathless strain of SS-1 were selected for further investigation. Most of these colonies contained cells that no longer formed visible sheaths, as determined by phase-contrast light microscopy and colloidal

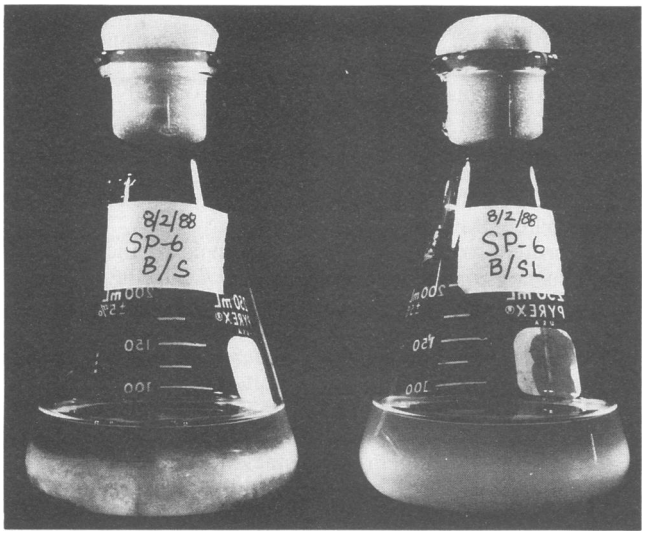

FIG. 1. Liquid cultures of *L. discophora* SP-6 (left) and its sheathless variant, SP-6(sl) (right). Both cultures were grown statically in MSVP liquid medium without added Mn^{2+} at 20°C for 48 h. The cultures were mixed just before the photograph was taken. SP-6 settled more rapidly to the bottom of the flask than did SP-6(sl) because of the presence of the sheath, which promoted floc formation.

iron staining. Strain SP-6(sl) was isolated from one such colony that arose on MSVP agar after transfer of SP-6 in PYG medium. SP-6(sl) grew in liquid media as small, dispersed aggregates, resulting in cultures that settled less rapidly than those of SP-6 (Fig. 1). SP-6(sl), like SS-1 (1, 2), maintained its ability to oxidize Mn^{2+} despite loss of its sheath-forming ability. Neither of the sheathless strains reverted to the sheath-forming phenotype when grown under conditions that maintained the sheath in SP-6.

The mean growth rate of both SP-6 and SP-6(sl) in MSVP and PYG liquid media with shaking at 150 rpm was 0.5 doubling · h^{-1} (Table 1). SP-6 grew at approximately the same rate (0.5 doubling · h^{-1}) in PYG medium and in a mineral salts-vitamin mixture with pyruvate, gluconate, or succinate added as a carbon energy source. Fumarate and malate were not utilized. Strain SS-1 grew faster in aerated PYG medium (0.7 doubling · h^{-1}) than did SP-6 (Table 1). This growth rate for SS-1 was comparable to the rate reported previously for SS-1 on PYG medium (2).

Taxonomic comparison of strains SP-6, SP-6(sl), and SS-1. Table 1 also summarizes the distinguishing characteristics of strains SP-6 and SS-1. Strain SP-6 was virtually identical to SP-6(sl), except for the absence of a sheath in the latter. SP-6 and SP-6(sl) differed slightly from SS-1; the growth rate of SS-1 was slightly faster than that of SP-6 in PYG medium, and its G+C content was slightly higher. Cells of all three strains showed similar cellular morphology (i.e., long, narrow rods with conspicuous poly-β-hydroxyalkanoate inclusions) (compare Fig. 2 with Fig. 1 in reference 2), a single polar flagellum, a preference for organic and carbon energy sources, and strictly aerobic growth. All strains excreted an Mn-oxidizing protein with an apparent molecular mass in the range of 105 to 110 kDa (Table 1).

Normalized SDS-PAGE gels containing SP-6 and SP-6(sl) membrane fractions containing similar amounts of protein were identical when stained with Coomassie blue. Both strains displayed major membrane protein bands at 88, 60 to 62, 38, and 35 kDa (Table 1). Approximately 10 minor bands also were resolved. Membrane protein profiles of SS-1 showed prominent bands at 84, 60, and 33 kDa (Table 1); no band was detected at the 38-kDa position. The SS-1 profile also lacked minor bands at 25 and 19 kDa that were present in the SP-6 and SP-6(sl) profiles. These results are similar to those reported by Willems et al. (29), who found nearly identical electrophoretogram protein patterns of whole cells of SP-6 and SP-6(sl); SS-1 showed overall similarities to the other two but clearly produced the most aberrant pattern (29).

Distribution of Mn oxides in the sheath. Light microscopic observation of SP-6(sl) cultures grown for 48 h in MSVP medium amended with 0.1 mM Mn^{2+} (Fig. 2a) showed that the Mn oxide flocs formed during growth were loosely associated with the sheathless filaments.

Similar results were reported previously for SS-1 cells growing in PYG medium amended with Mn^{2+} (compare Fig. 2a with Fig. 1b in reference 2). By contrast, when MSVP medium of SP-6 was amended with increasing amounts of Mn^{2+}, Mn oxide flocs were associated with the sheath at lower concentrations of Mn^{2+} (0.005 and 0.1 mM) (Fig. 2b and c), the sheath accumulated Mn oxide at discrete locations along its length, and it became progressively more visible in the phase-contrast microscope with increasing concentrations of Mn^{2+} (Fig. 2b and d). In MSVP medium amended with 0.2 mM Mn^{2+}, sheaths appeared as refractile, tubelike structures (Fig. 2d). Nonsheathed cells separated from sheathed filaments were observed regularly in all sheathed cultures (bottom Fig. 2c), which is consistent with the classical description of the life cycle of *Leptothrix* spp. and other sheathed bacteria in the *Leptothrix-Sphaerotilus*

TABLE 1. Comparison of *L. discophora* SS-1 and SP-6

Strain	Sheath formation	Cell diam (μm)	Flagellum	Poly-β-hydroxyalkanoate	Carbon source utilization[a]					Mn-oxidizing protein size (kDa)[b]	Catalase	Oxidase	Obligate aerobe	Growth rate (h^{-1})[c]	G+C content (mol %)[d]	Major membrane protein bands (kDa)[e]
					Pyr	Glu	Suc	Fum	Mal							
SS-1	−	0.6	Single, polar	+	+	+	+	−	−	105–110	+[f]	+	+	0.7	71.1	84, 60, 33
SP-6[g]	+	0.6	Single, polar	+	+	+	+	−	−	105–110	+[f]	+	+	0.5	69.4	88, 60–62, 38, 35

[a] Pyr, pyruvate; Glu, gluconate; Suc, succinate; Fum, fumarate; Mal, malate.
[b] Range of values found in this study and reported in references 3 and 7.
[c] In PYG with shaking at 150 rpm, 25°C.
[d] Data from reference 29.
[e] Membrane fractions containing equivalent amounts of protein were subjected to SDS-PAGE and stained with Coomassie blue as described in Materials and Methods.
[f] Very weak.
[g] Strain SP-6(sl) was identical to SP-6 except for the absence of sheath formation in SP-6(sl).

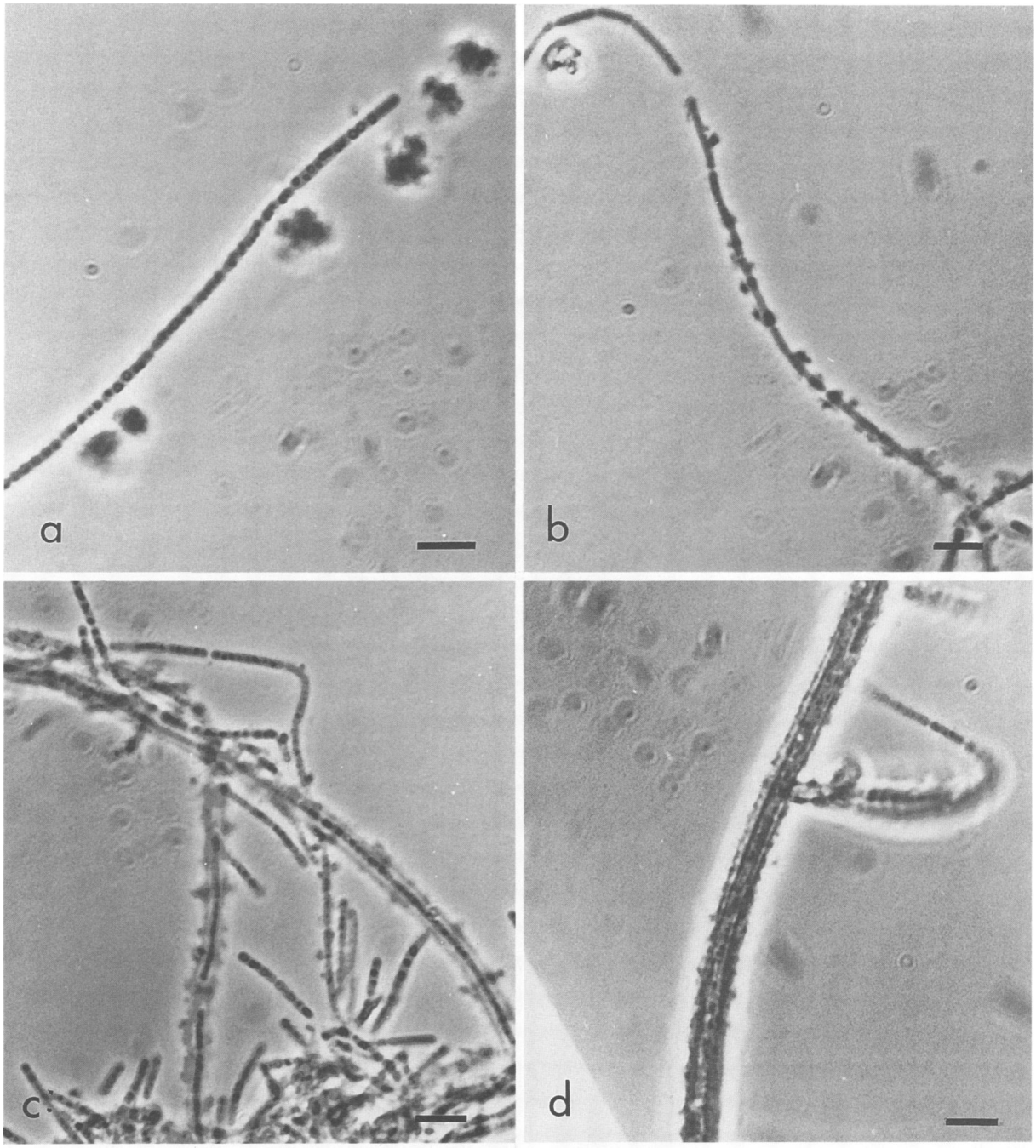

FIG. 2. Phase-contrast photomicrographs comparing the morphology of SP-6(sl) (a) with that of SP-6 (b, c, d) and showing the influence of increasing Mn^{2+} concentrations on the formation of Mn oxides associated with the sheath of SP-6. Both strains were grown statically at 25°C for 48 h in MSVP liquid medium with various amounts of Mn^{2+} added. (a) SP-6(sl), 0.1 mM Mn^{2+}. Dense Mn oxide particles were formed at some distance from cell filaments. (b) SP-6, 0.05 mM Mn^{2+}. Mn oxide particles formed at discrete locations along the filaments, but the sheath is not visible. (c) SP-6, 0.1 mM. Mn oxide particles formed at discrete locations along the sheath, and the sheath appears phase dense. (d) SP-6, 0.2 mM Mn^{2+}. Mn oxide encrusted the entire sheath, which appears refractile under phase-contrast optics. Bars, 5 μm.

group alternating between swarmer and sheathed cells (21, 28).

The ultrastructure of the sheath of SP-6 was best revealed in thin sections of cells grown without added Mn^{2+}. Electron microscopy showed that the sheath is a multilayered structure consisting of an electron-dense, 30- to 100-nm layer covered by a more diffuse capsular layer of variable thickness (Fig. 3a and b). The 30- to 100-nm layer is contacted by blebs of the outer membrane (Fig. 3b, arrows). In most cases, the cells had shrunk away from the sheath but the cell envelope remained attached to the inner sheath surface by the blebs. The blebs were seen in all sheathed cells as

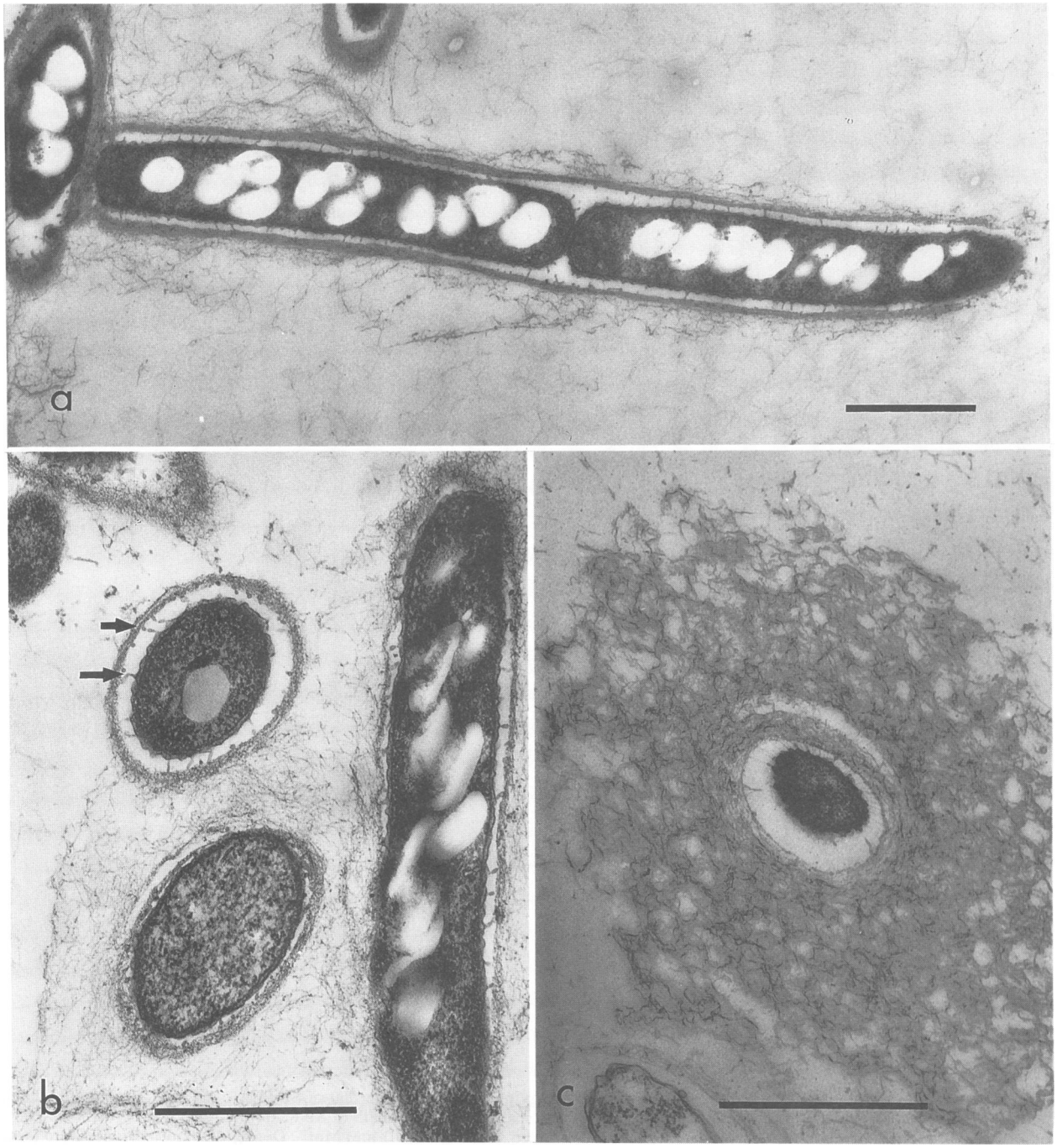

FIG. 3. Conventional transmission electron micrographs of SP-6 cells grown statically in MSVP liquid medium at 25°C for 48 h. The electron micrographs in panels a and b show longitudinal and cross sections, respectively, of SP-6 filaments grown without added Mn^{2+}. The principal sheath layer appears as an electron-dense 30- to 100-nm layer external to the cell envelope. The round, electron-transparent areas inside cells are poly-β-hydroxyalkanoate. The primary sheath layer has a discrete inner boundary contacted by extensions of the outer membrane of the cell envelope (arrows in panel b). Outside the primary sheath layer is a diffuse capsular zone of variable thickness containing fine, electron-dense filaments. The micrograph in panel c shows a cross section of an SP-6 filament grown in the presence of 0.2 mM Mn^{2+} (compare with the light micrograph in Fig. 2d). The Mn oxide-impregnated capsular layer is visible as a large zone of Mn oxide radiating outward from the primary sheath layer. Both layers appear to be uniformly encrusted with Mn oxides. Shrinkage of the cells during fixation and dehydration resulted in the empty space visible between the cell and the two sheath layers in the upper center of the micrograph. Bars, 1 μm.

electron-dense bridges or extensions of the outer membrane between the cell and inner sheath layer (Fig. 3). The capsular layer varied in thickness. It consisted of fibrous capsular material (Fig. 3a and b) that was less structured than the primary layer of the sheath. These results are similar to those of Beveridge (5), showing the same principal sheath and capsular layers in freeze-substituted SP-6 cells.

Thin sections of heavily encrusted sheaths showed that electron-dense Mn oxides were evenly distributed in both layers of the sheath matrix (Fig. 3c). The Mn oxide in SP-6 sheaths, like that associated with SS-1 exopolymers (2, 4) was poorly crystallized. It is similar in ultrastructural morphology to the Mn oxides associated with exopolymers of many other Fe and Mn oxide-depositing bacteria and fungi (13, 15). Most cells within Mn-encrusted sheaths also appeared to shrink during the fixation and dehydration procedure. The shrinkage sometimes caused breaks between the layers of Mn oxide encrusted sheaths (Fig. 3c).

STEM energy-dispersive X-ray analysis of sheathed cells confirmed the presence of significant amounts of Mn in the sheath (Fig. 4a and b). Small amounts of Mn were also detected inside some cells (data not shown); however, the internal Mn peaks were small in comparison with those of the sheath. They probably resulted from background X-rays arising from the higher concentrations of Mn oxides in the sheath. It is interesting that X-ray spectra characteristic of Fe were not detected in these sheaths, even though Fe was added to the growth medium at 1 ppm and Fe oxides would be expected to accumulate in the sheath. Apparently, the Fe concentration was reduced in the medium by cellular uptake to support growth, thus preventing Fe oxide accumulation in the sheath.

Approximately 10% of the cells examined by STEM contained electron-dense polyphosphate inclusions (Fig. 4a and c, arrows). Similar inclusions were also identified as polyphosphate in freeze-substituted SP-6 cells by Beveridge (6). Polyphosphate inclusions were not observed in a previous ultrastructural study of SS-1 cells (2). However, in that study, SS-1 was grown in PYG medium, in which the P_i supply is low relative to carbon and nitrogen. In the present work, we employed MSVP medium, which contained sufficient phosphate to cause accumulation of polyphosphate in storage granules, if not excess phosphate. Polyphosphate storage inclusions have not been reported previously in *Leptothrix* spp.

Distribution of Mn-oxidizing activity in fractionated cultures. In general, cultures of sheathless strains SS-1 and SP-6(sl) produced less total activity than did cultures of SP-6 at equivalent cell density but the sheathless strains excreted a higher proportion of their activity into the medium (Table 2). Approximately 66% of the Mn-oxidizing activity of SS-1 was found in the spent medium supernatant, compared with 57% for SP-6. Supernatants of SP-6(sl) were much more active than the complete culture from which they were derived. For unexplained reasons, the supernatants of SP-6(sl) contained approximately fourfold higher activity after centrifugation than before (Table 2). This phenomenon was never observed for SP-6 or SS-1, but it was very reproducible for SP-6(sl). The presence of SP-6(sl) cells could have inhibited the Mn-oxidizing activity in the medium; however, the mechanism of such inhibition is unexplained.

Sheathed cells of SP-6 retained a significant proportion of the original activity after washing in distilled water (18% in the experiment illustrated in Table 2), whereas sheathless cells showed no Mn-oxidizing activity after they were washed. When distilled-water-washed sheathed cells were subjected to EDTA–lysozyme–*N*-lauryl sarcosine treatment, cells were lysed and their remnants were removed (11) but the sheaths retained measurable Mn-oxidizing activity. Thus, the treatments involved in lysis and removal of cells from the sheaths also removed or inactivated much of the Mn-oxidizing activity; however, despite these harsh treat-

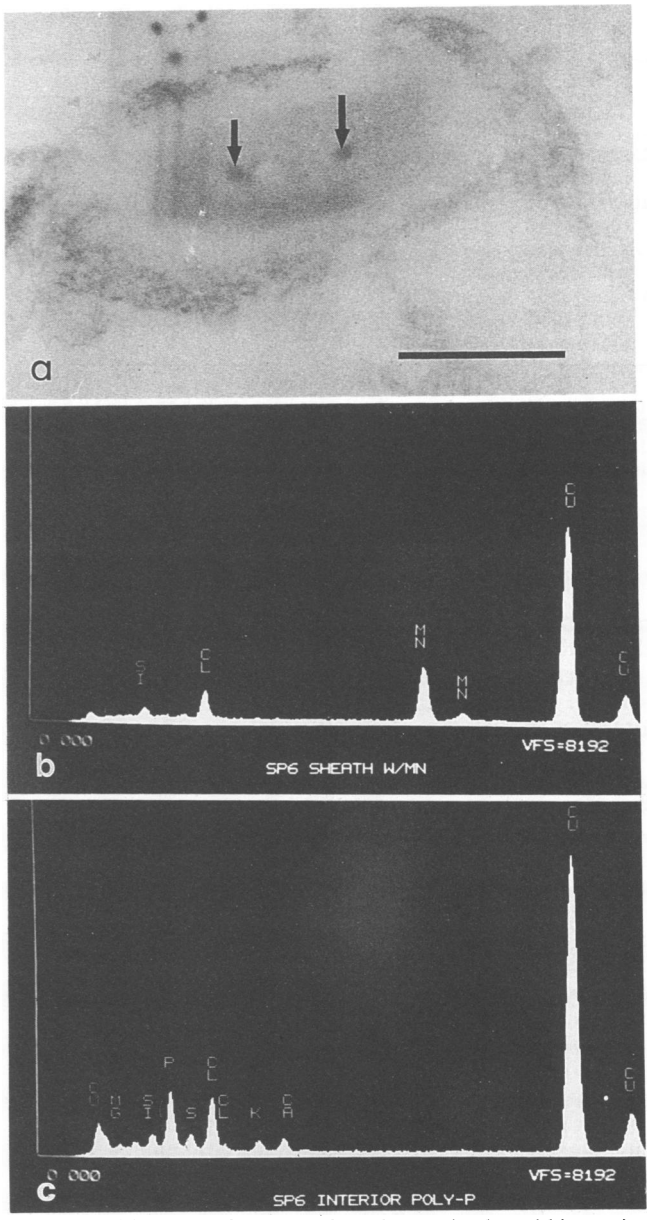

FIG. 4. (a) STEM of an unosmicated, unstained semithin section of an SP-6 filament. Growth conditions were as described in the legends to Fig. 2d and 3c. The low contrast of the cell and sheath resulted from omission of the usual contrasting agents from the fixation procedure. The arrows indicate polyphosphate inclusions. A sheath containing a lesser amount of Mn oxide was selected for energy-dispersive X-ray analysis to reduce Mn background signals. A large Mn peak is visible in the X-ray spectrum of the sheath (b), but no Mn was detected inside the cells (c). The phosphorus peaks in panel c confirm that the electron-dense bodies inside the cells (arrows in panel a) are polyphosphate. The large copper peaks at the right of each spectrum are from the specimen support grid; the chlorine peaks to the left arise from chlorine in the embedding resin.

TABLE 2. Recovery of Mn-oxidizing activity in fractions of *L. discophora* cultures[a]

Fraction	% Recovery		
	SP-6	SP-6(sl)	SS-1
Cell suspension in culture medium	100 (730)[b]	100 (90)	100 (71)
10,000 × *g* supernatant (unconcentrated)	57 (420)	400 (360)	66 (44)
Distilled-water-washed cells and sheaths	18 (132)	0 (0)	0 (0)
Washed cell-free sheaths (after EDTA-lysozyme-detergent treatment)	0.3 (4.5)	NA[c]	NA

[a] All cultures were grown to the early stationary phase (~10^8 cells · ml^{-1}) in 100 ml of MSVP medium without added Mn for 36 h at 25°C. SP-6 was a static culture. SP-6(sl) and SS-1 were shaken at 150 rpm. The data shown are derived from duplicate measurements of Mn-oxidizing activity in one experiment. Similar results were obtained in three other experiments.

[b] Total activity is given in parentheses as nanomoles of Mn oxidized per minute per milliliter × final fraction volume.

[c] NA, not applicable.

ments, some Mn-oxidizing activity remained tightly bound in the sheath.

Concentration and SDS-PAGE analysis of Mn-oxidizing activity. Mn-oxidizing activity was concentrated from the 10,000 × *g* supernatant of MSVP-grown SP-6 cultures by using a sequential ultrafiltration-dialysis procedure (Table 3) which resulted in an overall three- to fivefold increase in specific activity over the original culture activity. Compared with results reported earlier for SS-1 (3), the increase in specific activity was slightly higher, but a drastic loss of activity during the concentration procedure which occurred routinely in the previous work was much less severe in the present procedure. In the earlier work, only 1.2% of the original activity was recovered in the final concentration step, for about an 80-fold average loss of the original activity. In the procedure used for SP-6, the comparable loss of activity was about fourfold (Table 3).

SDS-PAGE showed that SP-6, SP-6(sl), and SS-1 all produced similar Mn-oxidizing protein bands that migrated to approximately the 108-kDa position on 12.5% polyacrylamide gels (Fig. 5). SP-6 concentrates contained a 108-kDa Coomassie blue-staining protein band (Fig. 5, lane a) and a doublet of Mn-oxidizing bands which migrated to approximately 108 and 103 kDa (Fig. 5, lane b). The latter values are comparable to the mean values of 110 and 106 kDa for the Mn-oxidizing doublet of SS-1 detected on 10% polyacrylamide gels (3). SP-6(sl) and SS-1 did not consistently show Coomassie blue-staining bands at the 108-kDa position in these experiments, suggesting that there was less Mn-oxidizing protein in these preparations.

Other properties of the Mn-oxidizing activity in concentrated culture supernatants of SP-6 also were similar to those found previously in SS-1 (3). Besides its stability in SDS and *N*-lauryl sarcosine, the Mn-oxidizing activity of SP-6 was found to be strongly inhibited by heating at 90 to 95°C for 10 min, treatment with 1 mM sodium azide, and exposure to Mn^{2+} concentrations above 250 μM.

DISCUSSION

A sheath-forming strain of *L. discophora* was isolated and found to maintain its sheath in MSVP medium under low-aeration conditions at temperatures between 20 and 25°C. Slow-growth conditions, combined with storage of the cultures on solid medium at 4°C, facilitated long-term maintenance of the sheath-forming cultures. Cultures of SP-6 have been maintained in our laboratory for 8 years. We have used the same cultural procedures to isolate and maintain similar sheath-forming strains of *L. discophora* from a natural iron seep near Ithaca, N.Y. Willems et al. (29) placed our *L. discophora* strains in a group within rRNA superfamily III with relatively high guanine-plus-cytosine contents. They showed that these strains are equidistantly related to *Sphaerotilus natans, Rhodocyclus gelatinosus, Pseudomonas saccharophila*, and *Alcaligenes latus* (29).

Sheathless strain SP-6(sl) was isolated by transferring SP-6 several times at 24-h intervals in well-aerated cultures under conditions that favored rapid growth. Sheathless strains were isolated in this manner from SP-6 cultures growing in either MSVP or PYG medium. Apparently, the more rapid growth rate promoted loss of the sheath independently of medium composition. These findings and those of others (15, 28) indicate that sheath-forming capacity is an unstable cultural trait of *L. discophora*. Neither SP-6(sl) nor SS-1 has ever reverted to the sheathed phenotype under the cultural conditions used to promote sheath maintenance in SP-6. These results suggest that an easily lost genetic element, such as a plasmid, may carry the genes that code for sheath formation. Preliminary evidence (11) suggested that SP-6 contains a single, large plasmid not present in SP-6(sl) or SS-1; however, this finding could not be confirmed in later work (27). Thus, the underlying genetic basis for loss of the sheath-forming phenotype remains unclear.

Until now, all biochemical studies on Mn and Fe oxidation by *L. discophora* have been done with sheathless strain SS-1 (3, 7, 9, 10). The lack of a sheath in this strain leaves open questions concerning the relationship between the Mn- and

TABLE 3. Concentration of Mn-oxidizing activity from *L. discophora* SP-6 cultures[a]

Fraction	Final vol (ml)	Total activity[b]	% Recovery	Total amt of protein (μg)	Sp act[c]	Fold increase in sp act
Cell suspension in culture medium	100	260	100	1,400	0.19	1.0
10,000 × *g* supernatant (unconcentrated)	96	168	65	192	0.88	4.6
Supernatant concentrated and washed by ultrafiltration	5.5	58	22	96	0.61	3.2
Ultrafiltered supernatant concentrated by dialysis in Aquacide IV	3.0	45	17	75	0.61	3.2

[a] Growth in MSVP medium without added Mn for 48 h at 25°C in static cultures. The data shown are derived from duplicate measurements of Mn-oxidizing activity in one experiment. Similar results were obtained in two other experiments.

[b] Nanomoles of Mn oxidized per minute per milliliter × final volume.

[c] Nanomoles of Mn oxidized per minute per milligram of protein.

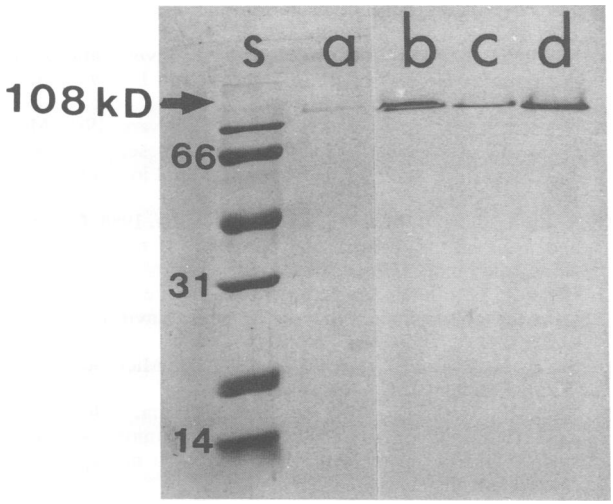

FIG. 5. Electrophoretogram of concentrated supernatants from SP-6 (lanes a and b), SP-6(sl) (lane c), and SS-1 (lane d). Protein standards (lane s) were Bio-Rad SDS-PAGE standards. Cultures were grown as described in Table 3, footnote *a*. The 10,000 × *g* supernatant from each culture was concentrated by the ultrafiltration-dialysis procedure, and aliquots of the final concentrates were subjected to SDS-PAGE. Lanes: a and b, 0.7 mg of total protein; c, 0.5 mg of total protein; d, 0.8 mg of total protein. After electrophoresis, the gel was bisected. The portion containing lanes s and a was stained with Coomassie blue; the portion containing lanes b, c, and d was washed in distilled water to remove SDS and then soaked in $MnSO_4$ solution to determine Mn-oxidizing activity (3). Note that the Coomassie blue-staining band at 108 kDa in lane a corresponds to the Mn-oxidizing band of SP-6 in lane b. In this experiment, no Coomassie blue-staining bands were detected at the 108-kDa position in lanes loaded with SP-6(sl) and SS-1 supernatants. However, the 108-kDa Mn-oxidizing bands (b, c, and d) were readily detected.

Fe-oxidizing proteins and the extracellular polymers produced by the organism (23). Does the sheath provide a matrix in which the Mn- and Fe-oxidizing proteins are concentrated? SP-6 cultures contained higher total Mn-oxidizing activity but excreted less of their Mn-oxidizing activity into the supernatant than did the sheathless strains (Table 2). It was possible to extract the activity from the sheath by washing with distilled, deionized water, indicating that the activity is associated with capsular polymers. However, even after treatment by an EDTA-lysozyme-detergent procedure to lyse and remove cells, a small amount of Mn-oxidizing activity remained in the sheaths, showing that the Mn-oxidizing protein is bound in the sheath matrix relatively tightly. This interpretation was supported by electron microscopy showing that the sheath consists of two layers in which Mn oxides are deposited (Fig. 3c). Apparently, the Mn-oxidizing protein is associated with sheath polymers, probably the capsular polymers which are found in both layers of the sheath.

Some aspects of SP-6 sheath chemistry have been studied further (11, 12, 14). This work shows that the sheath is a heteropolymer made up primarily of heteropolysaccharides and protein. The heteropolysaccharides contain a mixture of uronic acids and amino sugars. Staining with cationic colloidal iron indicates that they carry a net negative charge. The sheath proteins contain a high proportion of cysteine residues, and its matrix appears to be held together by extensive disulfide bonding (11, 12, 14). Both the negative charges and the sulfhydryl groups could act in scavenging metallic cations. We speculate that the anionic sheath polymers serve as scavengers of Mn^{2+} ions which are oxidized by the Mn-oxidizing protein bound in the sheath matrix. Mn oxidation occurs first on the outer capsular layer but eventually within the entire sheath matrix. At higher concentrations of Mn^{2+}, the Mn oxides themselves could serve to scavenge Mn^{2+}, resulting in autoxidation and further deposition of Mn oxides in the sheath. The net result is that eventually the entire organic matrix of the sheath becomes encrusted with Mn oxides, as shown in Fig. 3c.

Although we have not looked for an Fe-oxidizing protein in SP-6, the taxonomic similarities between SS-1 and SP-6 strongly indicate that a protein like that found in SS-1 (9, 10) is present in SP-6. Following the model outlined above, we can predict that the activity of the Fe-oxidizing protein would cause Fe(III) oxide to be distributed in the sheath matrix in a similar fashion.

Binding of Mn to extracellular polymers has been associated with Mn oxidation in a number of other microorganisms (22, 23). For example, it has been shown that budding bacteria deposit Mn oxides extracellularly in association with acidic polysaccharides (15, 18), and ultrastructural analysis of an Mn-oxidizing marine pseudomonad suggested that Mn binding and oxidation occur in its glycocalyx (23). We have shown recently that a basidiomycetous fungus produces exopolymer-containing Mn oxide particles with a morphology resembling that of the genus *Metallogenium* (13). The question of whether or not these extracellular metal-accumulating systems are related to the *L. discophora* system now arises. Further comparison of extracellular polymer-associated microbial Mn- and Fe-oxidizing systems is warranted.

ACKNOWLEDGMENTS

D. Emerson was supported by Hatch formula funds from USDA and NSF grant BSC-8617408.

We thank Maria Cochran for technical assistance. We are indebted to Carole Daugherty and Rhea Garen for help with the STEM energy-dispersive X-ray analysis. Terry Beveridge generously shared unpublished results with us. We are also grateful to Patti Lisk for expert secretarial assistance.

REFERENCES

1. **Adams, L. F., and W. C. Ghiorse.** 1985. Influence of manganese on growth of a sheathless strain of *Leptothrix discophora*. Appl. Environ. Microbiol. **49:**556–562.
2. **Adams, L. F., and W. C. Ghiorse.** 1986. Physiology and ultrastructure of *Leptothrix discophora* SS-1. Arch. Microbiol. **145:** 126–135.
3. **Adams, L. F., and W. C. Ghiorse.** 1987. Characterization of extracellular Mn^{2+}-oxidizing activity and isolation of an Mn^{2+}-oxidizing protein from *Leptothrix discophora* SS-1. J. Bacteriol. **169:**1279–1285.
4. **Adams, L. F., and W. C. Ghiorse.** 1988. Oxidation state of Mn in the Mn oxide produced by *Leptothrix discophora* SS-1. Geochim. Cosmochim. Acta **52:**2073–2076.
5. **Beveridge, T. J.** 1989. Metal ions and bacteria, p. 1–30. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, Inc., New York.
6. **Beveridge, T. J.** Personal communication.
7. **Boogerd, F. C., and J. P. M. DeVrind.** 1987. Manganese oxidation by *Leptothrix discophora*. J. Bacteriol. **169:**489–494.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
9. **Corstjens, P. L. A. M., J. P. M. DeVrind, P. Westbroek, and E. W. DeVrind-DeJong.** 1992. Enzymatic iron oxidation by *Leptothrix discophora*: identification of an iron-oxidizing protein. Appl. Environ. Microbiol. **58:**450–454.

10. **DeVrind-DeJong, E. W., P. L. A. M. Corstjens, E. S. Kempers, P. Westbroek, and J. P. M. DeVrind.** 1990. Oxidation of manganese and iron by *Leptothrix discophora*: use of *N,N,N'N'*-tetramethyl-*p*-phenylenediamine as an indicator of metal oxidation. Appl. Environ. Microbiol. **56:**3458–3462.
11. **Emerson, D.** 1989. Ph.D. thesis. Cornell University, Ithaca, New York.
12. **Emerson, D.** Unpublished data.
13. **Emerson, D., R. E. Garen, and W. C. Ghiorse.** 1989. Formation of Metallogenium-like structures by a manganese-oxidizing fungus. Arch. Microbiol. **151:**223–231.
14. **Emerson, D., and W. C. Ghiorse.** Unpublished data.
15. **Ghiorse, W. C.** 1984. Biology of iron- and manganese-depositing bacteria. Annu. Rev. Microbiol. **38:**515–550.
16. **Ghiorse, W. C., and S. D. Chapnick.** 1983. Metal depositing bacteria and the distribution of manganese and iron in swamp waters. Environ. Biogeochem. Ecol. Bull. **35:**367–376.
17. **Ghiorse, W. C., and H. L. Ehrlich.** 1992. Microbial biomineralization of iron and manganese. *In* R. W. Fitzpatrick and H. C. W. Skinner. Iron and manganese biomineralization processes in modern and ancient environments, in press. CATENA, Supplement 21. Cremlingen-Destedt, Germany.
18. **Ghiorse, W. C., and P. Hirsch.** 1979. An ultrastructural study of iron and manganese deposition associated with extracellular polymers of Pedomicrobium-like budding bacteria. Arch. Microbiol. **123:**213–226.
19. **Jung, W. K., and R. Schweisfurth.** 1979. Manganese oxidation by intracellular protein of a *Pseudomonas* species. Z. Allgemeine Mikrobiol. **19:**107–115.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Mulder, E. G., and W. L. van Veen.** 1963. Investigations on the *Sphaerotilus-Leptothrix* group. Antonie van Leeuwenhoek **29:** 121–153.
22. **Nealson, K. H., R. A. Rosson, and C. R. Myers.** 1989. Mechanisms of oxidation and reduction of manganese, p. 383–411. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, Inc., New York.
23. **Nealson, K. H., B. M. Tebo, and R. A. Rosson.** 1988. Occurrence and mechanisms of microbial oxidation of manganese. Adv. Appl. Microbiol. **33:**279–318.
24. **Ostle, A. G., and J. G. Holt.** 1982. Nile blue A as fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. **44:**238–241.
25. **Seeley, H. W., and P. J. VanDemark.** 1981. Microbes in action. W. H. Freeman & Co., San Francisco.
26. **Seno, S., T. Tsujii, T. Ono, and S. Ukita.** 1983. Cationic cacodylate iron colloid for the detection of anionic sites on cell surface and the histochemical stain of acid mucopolysaccharides. Histochemistry **78:**27–31.
27. **Siering, P., and W. C. Ghiorse.** Unpublished data.
28. **van Veen, W. L., E. G. Mulder, and M. H. Deinema.** 1978. The *Sphaerotilus-Leptothrix* group of bacteria. Microbiol. Rev. **42:** 329–356.
29. **Willems, A., M. Gillis, and J. De Ley.** 1991. Transfer of *Rhodocyclus gelatinosus* to *Rubrivivax gelatinosus* gen. nov., comb. nov., and phylogenetic relationships with *Leptothrix, Sphaerotilus natans, Pseudomonas saccharophila*, and *Alcaligenes latus*. Int. J. Syst. Bacteriol. **41:**65–73.